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Angiogenesis in Normal Tissue Adjacent to Colon Cancer

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Background and Objectives: Angiogenesis in malignant neoplasms, as measured by microvessel density, has been shown to correlate with survival or stage in some studies of breast, gastric, and colorectal cancer. We hypothesized that aggressive cancers promote angiogenesis in normal tissue adjacent to the invading neoplasm.

Methods: To test this hypothesis, 36 specimens of colon adenocarcinoma curatively resected between 1986 and 1990 were sectioned and stained for factor VIII-related antigen, vascular endothelial growth factor (VEGF), and interleukin-8 (IL-8). Microvessel density was measured within the colon cancer and in adjacent, histologically normal tissue. Clinical/pathological variables were examined using multivariate analysis and Student *t*-test.

Results: Microvessel density was higher in the neoplasms (26.0 ± 1.66/0.25 mm^2) than in the surrounding normal tissue (22.3 ± 1.88/0.25 mm^2) ($P = 0.03$). The difference was primarily due to smaller neoplasms (T1 and T2) which had vessel counts of 10.6 ± 0.74/0.25 mm^2 in the adjacent normal tissue compared to vessel counts of 18.9 ± 3.02/0.25 mm^2 within these tumors ($P = 0.02$). T3 and T4 neoplasms had equivalent amounts of angiogenesis within the lesion (26.9 ± 1.81/0.25 mm^2) and in the histologically normal margin (24.2 ± 1.98/0.25 mm^2) ($P = 0.12$). VEGF was present in the tumor microenvironment in 100% and IL-8 in 45% of specimens stained for these angiogenic cytokines. Microvessel density did not correlate with 5-year survival.

Conclusions: Our data suggest that colon cancers that invade through the muscularis propria may have a greater ability to induce angiogenesis in adjacent normal tissue. *J. Surg. Oncol. 1998;69:230–234.*

KEY WORDS: angiogenesis; vascular endothelial growth factor; interleukin-8; colon cancer

INTRODUCTION

Tumor angiogenesis has been implicated as a key factor in tumor growth and metastasis. Angiogenesis is thought to be controlled by chemical signals known as angiogenic factors such as vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8). Angiogenesis in a solid tumor may be described as the result of a paracrine relationship between tumor cells and host cells [1] via a wide variety of chemical signaling systems including angiogenic factors. A neoplasm capable of inducing neovascularization stimulates host endothelial cells to proliferate, migrate, and secrete proteases [2]. These proteases erode tumor stroma and facilitate vascular growth into the tumor, which in turn provides vascular access to the systemic circulation for neoplastic cells. The invad-

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TABLE I. Patient Demographics and Tumor Characteristics of 36 Patients Studied for Angiogenesis in Normal Tissue Adjacent to Colon Cancer

Parameter	Mean ± SE
Patient age (years)	68 ± 2
Sex (M/F)	14/22
Survival (months)	37.4 ± 3.5
Mean tumor size (cm)	4.56 ± 0.36
Node positive (%)	36
Proximal colon (%)	38.9
Distal colon (%)	61.1
T1	2
T2	3
T3	23
T4	8

ing vasculature and associated cells stimulate proliferation of neoplastic cells which further advances the neoplasm into the vascular phase of its life cycle [2].

It seems reasonable to expect that the quantity of vessels in a tumor may correlate with clinical outcome. Although some studies have failed to demonstrate a correlation between tumor microvessel density and cancer-specific survival [3–5], others have found such a correlation, especially when analysis is confined to node-negative cancers [6,7].

The influence of tumor angiogenesis on adjacent normal tissue is only beginning to be examined. In this report, we describe our observations of angiogenesis in colon cancer and the normal tissue immediately adjacent to the primary colon tumor and correlate these findings with clinical parameters and survival.

MATERIALS AND METHODS

The records of 36 patients with colon adenocarcinoma curatively resected at the University of Connecticut School of Medicine between 1986 and 1990, with 5-year follow-up, were retrieved. Patient data (sex, age, and survival) and tumor characteristics (location, size, depth of penetration, and nodal status) were collected from the tumor registry (Table I).

Paraffin-embedded tissue was sectioned and stained immunohistochemically for factor VIII-related antigen (Dako Polyclonal, Dako, Santa Barbara, CA) to visualize endothelial cells (Fig. 1). In addition, 10 tumors were immunostained for VEGF (Fig. 2) and IL-8 (Fig. 3).

Briefly, tissue sections of primary tumor were mounted onto slides, deparaffinized in xylene, and rehydrated in graded ethanol solutions (100%, 95%, 75%, 50%). Endogenous peroxidase activity was quenched by dipping the slides into a 0.0225% hydrogen peroxide methanol solution for 20 min. Slides were then air dried and incubated at room temperature in a blocking solution (5% normal goat serum in phosphate buffer) for 1 hr. Primary antibody was applied and the tissue was allowed

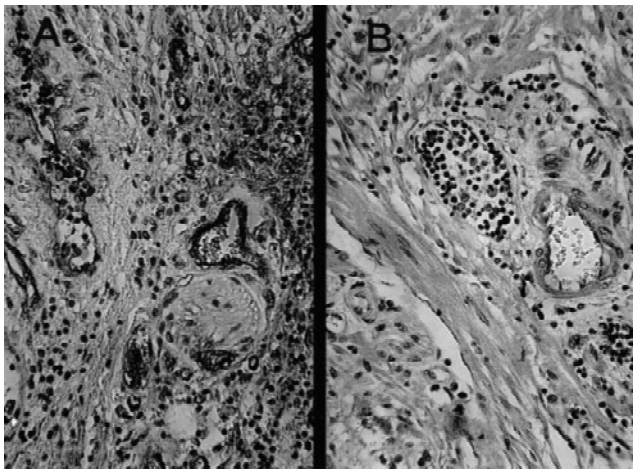

Fig. 1. **A:** Section of colon cancer tissue immunostained with monoclonal antibodies to factor VIII-related antigen. Vessels are indicated by stained endothelium, a visible lumen, and the presence of erythrocytes. **B:** Section of colon cancer tissue (control for A). ×200.

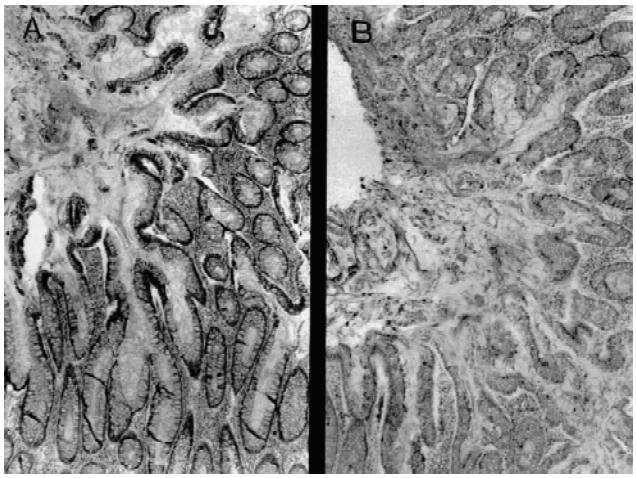

Fig. 2. **A:** Section of colon cancer tissue immunostained with monoclonal antibodies to VEGF. In this sample, VEGF was localized to the basal aspect of tumor cells. **B:** Section of colon cancer tissue (control for A). ×200.

to sit at 4°C overnight. The following day, slides were rinsed in phosphate buffer 3 times and treated with secondary antibody (Vector, Burlingame, CA) at 1/200 dilution for 1 hr and then rinsed 3 times in phosphate buffer solution. The tissue was then complexed with horseradish peroxidase streptavidin (Zymed, San Francisco, CA) at 1/100 dilution in phosphate buffer for 45 min at room temperature followed by development in 3-amino-9-ethylcarbazole (0.1 M sodium acetate buffer, pH 5, and 0.03% H_2O_2) solution for 10–20 min at room temperature. The tissue was then counterstained with hematoxylin. Finally, slides were washed in distilled water and dipped in dilute ammonium hydroxide and mounted in crystal mounting solution (Biomeda, Foster City, CA).

Slides stained with antibodies to factor VIII-related

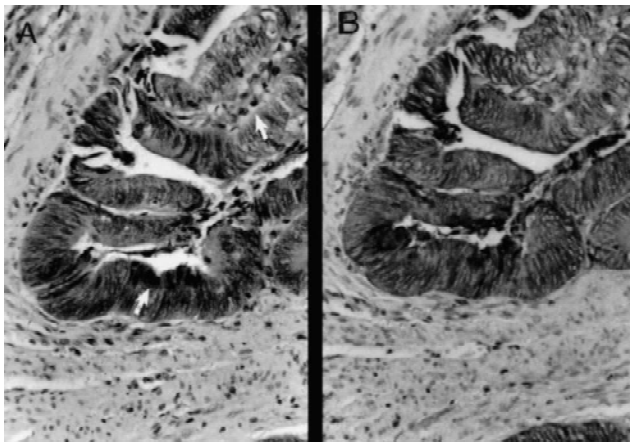

Fig. 3. **A:** Section of colon cancer tissue immunostained with monoclonal antibodies to IL-8. The presence of IL-8 was inconsistent; arrows point to positive staining in the tumor and stromal cells. **B:** Section of colon cancer tissue (control for A). ×200.

antigen were examined at low power to identify angiogenic ''hot spots'' within the tumor [7]. Hot spots were areas within the neoplasm which had apparent high vessel counts on low power. Vessels in 4 random ''hot spots'' within the invading margin of the tumor were counted and averaged. Microvessel density was quantified in these hot spots with an ocular micrometer grid (0.5 × 0.5 mm) under ×200 magnification. Vessels were identified based on the presence of endothelial staining and a visible lumen. Four random areas of non-neoplastic tissue adjacent to the invading margin and within ×200 view were also assessed for microvessel density and averaged. Each tumor had, therefore, two microvessel density counts: intratumoral and in the adjacent non-neoplastic tissue.

Statistical analysis was performed with SPSS software (SPSS Inc., Chicago, IL). Student *t*-test was used to compare the means of microvessel density. Data are presented as the mean ± standard error (SE). Kaplan-Meier survival curves plotted outcome in which the patient population was arbitrarily divided into a high microvessel and a low microvessel density group. To create these groups, microvessel density values were arranged into ascending order and the population was divided at the median value. The high vessel density group comprised those patients above the median value, while those below comprised the low vessel density group. Cox proportional hazards regression examined the relationship between patient data, tumor characteristics and 5-year outcome. $P < 0.05$ was considered significant.

RESULTS

Microvessel density was greater in the tumor (26.0 ± 1.66/0.25 mm^2) than in adjacent normal tissue (22.3 ± 1.88/0.25 mm^2) ($P = 0.03$). This difference was primarily found in earlier stage primary tumors (T1/T2) which had a tumor microvessel density of 18.9 ± 3.02/0.25 mm^2 compared to a microvessel density of 10.6 ± 0.74/0.25 mm^2 in adjacent normal tissue ($P = 0.02$). Advanced stage primary tumors (T3/T4) had the same microvessel density in both the tumor tissue (26.9 ± 1.81/0.25 mm^2) and adjacent normal tissue (24.2 ± 1.98/0.25 mm^2) ($P = 0.12$) (Table II).

TABLE II. Microvessel Density (Vessels/0.25 mm^2) at the Invasive Margin of Colon Adenocarcinoma and Normal Adjacent Tissue With Tumors Categorized by Depth of Invasion

		Mean ± SE		
	n	Tumor	Normal Tissue	*P*
T1/T2	5	18.9 ± 3.02	10.6 ± 0.74	0.02
T3/T4	31	26.9 ± 1.81	24.2 ± 1.98	0.12*

*Not significant.

Tumor cells, endothelial cells, and epithelial cells in the adjacent normal tissue all stained for the angiogenic cytokine VEGF in every specimen examined (Fig. 2). Interestingly, tumor stromal cells were not stained. Nevertheless, there was no significant correlation between microvessel density and staining intensity in either location. IL-8 production was not as ubiquitous. Tumor and endothelial cells were stained in only 5/11 specimens. In these cases, there was also some staining of adjacent epithelial cells (Fig. 3). No correlation was found between IL-8 staining and microvessel density.

In multivariate analysis, nodal status correlated with 5-year survival ($P = 0.02$), but microvessel density did not. Division of patients into high and low microvessel density groups failed to demonstrate a relationship of microvessel density in either tumor or adjacent normal tissue with survival (Figs. 4, 5).

DISCUSSION

Our finding that microvessel density is greater in colon cancers than in surrounding normal tissue is novel but expected and consistent with observations that the fraction of cycling endothelial cells is higher in the primary tumor than in adjacent mucosa [8]. The intriguing observation of this study is that larger, more invasive tumors have a greater influence on angiogenesis in nearby normal tissue than do smaller tumors. It supports the intuitively appealing concept that larger tumors diffuse more angiogenic cytokines into the immediately surrounding environment. Which diffusible cytokines might be responsible for induction of angiogenesis in normal tissue surrounding colon cancer? The list is long and their interactions complex [9,10], but VEGF is probably one of them. This cytokine induces the growth of leaky new blood vessels which results in an early matrix consisting of other angiogenic plasma proteins such as fibrin. VEGF may be induced by other angiogenic cytokines [11–13]

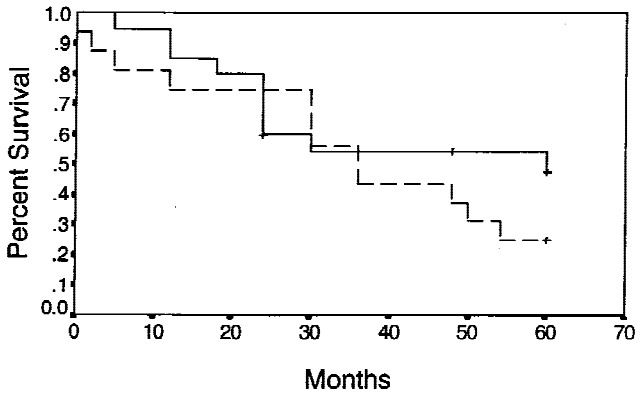

Fig. 4. Kaplan-Meier survival curves comparing the effect of tumor microvessel density between high vessel density (>22 vessels/0.25 mm^2; —) and low vessel density (<22 vessels/0.25 mm^2; ---) groups ($P = 0.29$).

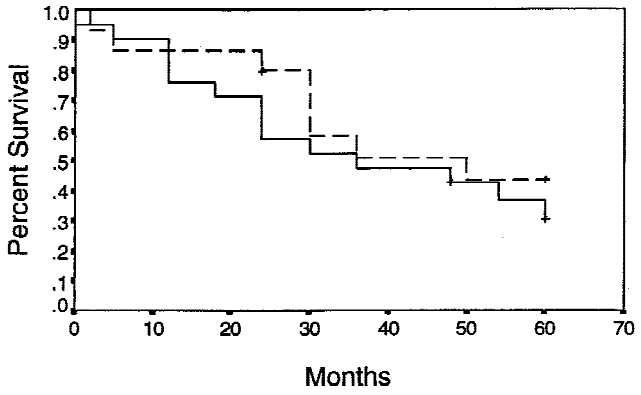

Fig. 5. Kaplan-Meier survival curves comparing the effect of microvessel density in normal tissue adjacent to colon cancer between high vessel density (>18 vessels/0.25 mm^2; —) and low vessel density (<18 vessels/0.25 mm^2; ---) groups ($P = 0.42$).

and may require still others such as basic fibroblast growth factor (bFGF) to induce angiogenesis [14], but its expression is ubiquitous when new tissue is being generated [15]. VEGF has been found in human tumors and its expression usually correlates with microvessel density [16,17]. Expression in human colon cancer particularly has been shown to correlate with microvessel density, metastasis, and survival [18]. Although we did not find such a correlation in this study, we found VEGF by immunohistochemical staining in tumor and adjacent normal epithelial cells in every specimen examined. IL-8 is another angiogenic cytokine that appears to play an important role in some tumors. We did not find it in every specimen examined in this study, although it has been found in head and neck [19], breast (Kurtzman et al., unpublished data), and prostate cancer [11] in our laboratory. Increased IL-8 production can increase invasion, angiogenesis, and metastasis in an experimental model of human melanoma [20]. The role of IL-8 in induction of neovascularization in colon cancer, however, is less clear, and based on our findings, is likely to be less crucial than the role of VEGF.

Microvessel density in the tumor and surrounding normal tissue did not correlate with prognosis. There are several techniques for quantifying blood vessels in tumors [21], and the selection of angiogenic hot spots is somewhat arbitrary. Thus, the determination of microvessel density may not be as broadly straightforward as other histologic parameters [22,23]. Since others have found that microvessel density in a number of tumors does predict metastatic behavior [6,16,24–28], we expect that a larger sample size in this study would have demonstrated an association with outcome. It may be, however, that microvessel density is not as powerful a prognostic indicator as nodal status, which did correlate with survival in this study.

CONCLUSIONS

Our data suggest that colon adenocarcinomas that penetrate the muscularis propria may have a greater ability to induce neovascularization in adjacent normal tissue than tumors which do not.

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